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Interleukin-12 induces a Th1-like response to *Burkholderia mallei* and limited protection in BALB/c mice

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This paper is dedicated to the memory of Tran C. Chanh.

Abstract

We evaluated the effect of interleukin (IL)-12 on the immune response to *Burkholderia mallei* in BALB/c mice. Mice were vaccinated with non-viable *B. mallei* cells with or without IL-12. There was a seven- to nine-fold increase in IgG2a levels, and a significant increase in the proliferative response and interferon (IFN)-γ production by splenocytes from mice that received *B. mallei* and IL-12. We saw an increase in survivors in the groups of mice that received *B. mallei* and IL-12 when challenged, compared to mice that received only *B. mallei* or IL-12. The results suggest that IL-12 can enhance the Th1-like immune response to *B. mallei* and mediate limited protection from a lethal challenge.

Keywords: IL-12; Th1 response; Limited protection

1. Introduction

Burkholderia mallei is a Gram-negative, non-motile bacillus, which causes glanders primarily in horses, mules, and donkeys. In the natural host infection by the oral route is the primary route and by close contact with infected animals. Infection in horses is more often manifested as a slow progressive, chronic disease, whereas in donkeys, the disease usually takes an acute course of infection with death occurring within a week to 10 days [1,2]. Early symptoms in the host may include thirst, fever, shivering, drooping of the head, tachycardia, weight loss, prostration, and malaise. Involvement of the lungs and upper respiratory tract is common and is manifested by a regional or diffuse pneumonia or pleuritis, and a nasal exudate, which is infectious. In infections by the cutaneous route, regional lymphnodes become enlarged and indurated (farcy), and may rupture and suppurate. Infections that originate by the oral or cutaneous route can develop into an acute septicemia after the primary infection and can quickly lead to death. There is no effective treatment for glanders in the natural host, and animals diagnosed with glanders are isolated and destroyed. In humans infection by glanders may be through the oral, nasal, ocular, or cutaneous routes. Progression of the clinical symptoms is similar to those in the natural host and may be presented as an acute localized or pulmonary form, or septicemic fatal illness. Also, a chronic lymphangitis and regional adenopathy may be seen. In humans, however, usually a combination of symptoms is observed. The relative ease of infection by the aerosol route was demonstrated in the laboratory a number of years ago [3], and by the cutaneous route just recently [4,5]. Presently, there is no vaccine, so it is important to develop a vaccine for this easily disseminated and infectious agent. We recently evaluated the murine immune response to non-viable *B. mallei* in BALB/c mice and found that a mixed T-helper 1 (Th1)-/T-helper 2 (Th2)-like cytokine response was obtained with a Th2-like subclass immunoglobulin (Ig) response [6]. Very little IL-12 and small but significant amounts of interferon (IFN)-γ were expressed, as well as small but significant

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14. ABSTRACT

We evaluated the effect of interleukin (IL)-12 on the immune response to Burkholderia mallei in BALB/c mice. Mice were vaccinated with non-viable B. mallei cells with or without IL-12. There was a seven- to nine-fold increase in IgG2a levels, and a significant increase in the proliferative response and interferon (IFN)-gamma production by splenocytes from mice that received B. mallei and IL-12. We saw an increase in survivors in the groups of mice that received B. mallei and IL-12 when challenged, compared to mice that received only B. mallei or IL-12. The results suggest that IL-12 can enhance the Th1-like immune response to B. mallei and mediate limited protection from a lethal challenge.

15. SUBJECT TERMS

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amounts of interleukin (IL)-4 and IL-10 were expressed by stimulated splenocytes obtained from mice vaccinated with non-viable *B. mallei* cells. Non-viable *B. mallei* preparations did not protect mice against a lethal challenge.

Because BALB/c mice were not protected by inactivated *B. mallei* preparations, we decided to evaluate the cytokine IL-12 as an adjuvant on the murine immune response to non-viable *B. mallei* cells. IL-12 can act as an intermediary signal between the innate and acquired immune response to the presence of a pathogen [7]. The expression of IL-12 is induced by the interaction of monocyte/macrophages, neutrophils, or dendritic cells with the invading pathogen, in part through the recognition by these cells of pathogen associated molecular patterns [8]. IL-12 can induce the expression of IFN-γ predominately by T-lymphocytes and NK cells and increase cytotoxic activity. The induction of a Th1-like immune response is crucial to the ability of the host to enhance the cellular immune response to limit the establishment of bacterial, protozoan, or fungal pathogens [9]. The requirement of IL-12 to increase resistance to infection was shown by the neutralization of IL-12 in a number of animal models [10–18]. Enhancement of resistance to infection was shown in other studies by the introduction of IL-12 before or after vaccination or infection [19–26]. Finally, in IL-12-deficient mice, it was shown that IL-12 is required to maintain a long-term, Th1-like immune response to intracellular protozoans [27–30].

2. Materials and methods

2.1. Bacterial strain and mice

B. mallei ATCC 23344 was grown and prepared as previously reported [6]. Briefly, a suspension of a glycerol stock was inoculated into 4% glycerol–1% tryptone (Difco, Becton Dickinson, Sparks, MD) broth (GTB) and grown overnight at 37 °C with shaking (250 rpm). After 20–24 h of growth, the cells were pelleted by centrifugation and suspended in sterile Hanks' balanced salt solution (HBSS). Non-viable cells were prepared by exposure to 2.1 megarads of gamma radiation. Sterility of all inactivated cell preparations was verified by plating an aliquot on GTB agar (Difco) plates. After inactivation, the cells were washed three times with HBSS and suspended to an appropriate dilution with sterile water. Female, 6- to 8-week-old, BALB/c mice were obtained from the National Cancer Institute (Frederick, MD).

2.2. Vaccinations and challenges

BALB/c mice in groups of 14–15 were vaccinated subcutaneously with 50 μg (equivalent to 1.3×10^7 colony forming units (CFU)) of non-viable *B. mallei* cells mixed with 100 μg of Alhydrogel (Brenntag Biosector A/S, Frederikssund, Denmark) [6,31] in a total volume of 0.2 ml. Different amounts of murine IL-12 (0–1.0 μg), a generous gift from Wyeth Research (formerly Genetics Institute, Cambridge, MA), were included with the non-viable cell-Alhydrogel mixture. Control mice that did not received any *B. mallei* or IL-12, or only IL-12 was vaccinated with the same amount of Alhydrogel (100 μg) used in the study group. All mice, including controls, were vaccinated twice, 3 weeks apart, and 3 weeks after the second boost [32], and 10 mice from each group, including from the controls, were challenged i.p. with 1×10^8 CFU in 0.5 ml of *B. mallei* and then observed for up to 21 days for survival [33]. The challenge dose was 143 50% lethal doses (LD_{50}), where 1 LD_{50} is 7×10^5 CFU for 6- to 8-week-old BALB/c mice [33].

Research was conducted in compliance with the Animal Welfare Act and other federal statues and regulations relating to animals and experiments involving animals and adheres to principles stated in the *Guide for the Care and Use of Laboratory Animals*, National Research Council, 1996. The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

2.3. Spleen cell cultures

Spleen cell cultures were prepared 3 weeks after the second vaccination boost as previously described [6,34]. Briefly, two or three spleens from each group were combined into 3 ml of wash medium [RPMI 1640 medium (Invitrogen Corp, Carlsbad, CA) containing 25 mM HEPES, 2 mM glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml)], and spleen cells were disaggregated with the end of a sterile 5 ml syringe handle. After allowing the large particles to settle, the supernatant was placed into a 15 ml conical tube and solution brought up to 12–14 ml with wash buffer. The tube was centrifuged at 1200 rpm for 10 min at room temperature, and the supernatant was discarded. The cells were suspended in 0.16 M ammonium chloride in 0.01 M Tris–HCl buffer (pH 7.5) to lyse the red cells, and wash buffer was added to stop the reaction. The cells were washed twice and suspended in RPMI 1640 medium (Invitrogen Corp.) containing 10% fetal calf serum, 25 mM HEPES, 2 mM glutamine, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 50 μM 2-mercaptoethanol, penicillin (100 U/ml), and streptomycin (100 μg/ml) at a cell concentration of 10×10^6 cells/ml.

2.4. Proliferation assay

To determine the proliferative response of splenocytes from mice vaccinated with *B. mallei* and IL-12, a 50 μl aliquot of a splenocyte suspension (2×10^6 cells/ml) was incubated in a 96-well plate in triplicate with and without non-viable *B. mallei* cells (10 μg/ml) (equivalent to 3.3×10^6 CFU) as previously described [6]. Concanavalin A (ConA) (5 μg/ml) was included as a control in the assay although the results are not shown. Duplicate cultures were prepared for each group of mice. After incubation for approximate 48 h at 37 °C with 5% CO_2, 1.0 μCi of [^{3}H]thymidine (TdR) at a specific

activity of 5 Ci/mmol (Amersham Life Sciences, Arlington Heights, IL) was added to each well and incubation continued overnight. Cells were harvested after 18 h [35,36], and the proliferative response was reported as the amount of [^{3}H]TdR incorporated ± standard deviation (S.D.). Since there were two samples for each group of mice and each sample was read in triplicate, the amount of [^{3}H]TdR incorporated for each group was reported as the mean of six readings.

2.5. Cytokine assays

Cytokine expressed in the supernatant of restimulated splenocyte cultures were determined after approximately 45–48 h of incubation as previously described [6]. Splenocyte cultures were prepared in duplicate from each group of vaccinated mice. Splenocyte cultures were incubated at 5×10^6 cells/ml in 24-well culture dishes with and without non-viable *B. mallei* cells (10 μg/ml)(equivalent to 3.3×10^6 CFU) at 37 °C with 5% CO_2. The amount of cytokine expressed was determined by a cytokine capture, enzyme-linked immunosorbent assay (ELISA) obtained from BD Pharmingen, (San Diego, CA) and each sample determined in triplicate. The results were reported as the mean concentration (picograms) expressed ± 1 S.D. Since there were two samples from each group of mice, and each sample was set up in duplicate, and the amount of cytokine expressed was performed in triplicate, the results were reported as the mean of 12 sample readings. The sensitivity of the cytokine ELISAs was 7.5–15 pg/ml.

2.6. Antibody assays

Antibody titers were determined by ELISA as described previously [6]. Two-fold dilutions of antisera were made in triplicate in 96-well, Immulon 2HB plates (Thermo Electron, Milford, MA), which previously contained 50 μl of non-viable, irradiated *B. mallei* cells (10 μg/ml in 0.1 M carbonate buffer, pH 9.5). Class or subclass Ig was determined with anti-Ig-horseradish peroxidase conjugate (Southern Biotechnology Associates (Birmingham, AL). The results are reported as the geometric mean of the reciprocal of the highest dilution giving a mean OD of at least 0.1, which is at least twice the background, and the standard error (S.E.).

2.7. Statistics

Titer was compared between groups by ANOVA with post hoc Dunnett's test. Survival rate was compared by Fisher exact tests with Bonferroni adjustment. Mean time to death was compared by *t*-test with Bonferroni adjustment. Survival curves were calculated by Kaplan Meier survival analysis with log-rank tests between groups. Probit analysis was used to assess effect of titer on survival. $P \leq 0.05$ was considered significant. All statistical analyses were conducted by SAS Version 9.1.

3. Results

3.1. Enhancement of the murine antibody response to non-viable B. mallei by IL-12

We examined the effect of varying amounts of IL-12 on the murine antibody response to non-viable *B. mallei*. Mice were vaccinated subcutaneously (s.c.) with 50 μg of non-viable *B. mallei* (groups 2–6), and mice in groups 3–6 also received 0.1–1.0 μg of IL-12, while mice in groups 7 and 8 received only 0.25 or 1.0 μg of IL-12, respectively (Table 1). All mice in the study received Alhydrogel as an adjuvant and were vaccinated twice. There was a good IgG and IgM immune responses to the *B. mallei* cells without IL-12 (Table 1, group 2) as we previously noted [3]. Adding 0.25 μg of IL-12 with *B. mallei* cells boosted the IgG titer almost two-fold, and the titer remained at the enhanced level with 0.5–1.0 μg of IL-12 (Table 1, groups 5 and 6). The IgM titer at the same time varied from one-half to twice the titer as obtained with only *B. mallei* cells. We were also able to detect *B. mallei*-specific IgA antibodies in mice from groups 4 and 6, which received 0.25 and 1.0 μg of IL-12, respectively (data not shown). Mice

Table 1
The murine immune response to non-viable *B. mallei* is differentially enhanced by IL-12

Group	Treatment[a]	Antibody titer[b]				Ratio
		IgG	IgM	IgG1	IgG2a	IgG2a/IgG1
1	Alhydrogel	<50	696 (1.15)	100 (1.00)	76 (1.19)	0.76
2	Bm	1280000 (1.25)	40000 (1.25)	3880235 (1.19)	211121 (1.60)	0.05
3	Bm + 0.01 μg IL-12	1940117 (1.68)	40000 (1.00)	6755881 (1.32)	735167 (1.82)	0.11
4	Bm + 0.25 ug IL-12	2560000 (1.86)	80000 (1.46)	3377941 (1.32)	1940117 (1.68)*,[c]	0.57
5	Bm + 0.50 ug IL-12	2228610 (1.30)	22974 (1.50)	3532108 (1.35)	1470334 (1.58)*	0.42
6	Bm + 1.00 ug IL-12	2228610 (1.50)	40000 (1.46)	1280000 (1.46)	1470334 (1.50)*	1.15
7	0.25 ug IL-12	200 (2.00)	283 (1.20)	50 (1.00)	200 (4.00)	ND[d]
8	1.00 ug IL-12	71 (1.20)	476 (1.20)	50 (1.00)	<50	ND

[a] All groups have Alhydrogel included. Bm is non-viable *Burkholderia mallei*.
[b] Data are geometric mean (±S.E.). A titer of 50 or less was considered negative. N is 5 for groups 1–6, and 4 for groups 7 and 8.
[c] When compared to *B. mallei* (group 2), * is $P < 0.05$.
[d] ND is not determined.

that received only IL-12 (Table 1, groups 7 and 8) gave non-specific background readings to *B. mallei*.

We also examined the levels of IgG1 and IgG2a subclass response to *B. mallei* as representing a Th2- or Th1-like immune response, respectively. Without IL-12, there was a 18-fold greater amount of anti-*B. mallei* IgG1compared to the amount of anti-*B. mallei* IgG2a produced (Table 1, group 2). Adding IL-12 with the *B. mallei* cells significantly increased IgG2a levels (Table 1, group 4–6; $P < 0.05$), until almost an equal amount of the two Ig subclasses were produced with the highest amount of IL-12 used. The relationship between the levels of IgG1 and IgG2a antibody levels with increasing amounts of IL-12 with the *B. mallei* cells can be seen by the change in ratio of IgG2a to IgG1 (shown in the last column in Table 1). Our results show that the murine immune response to *B. mallei* cells can be enhanced by IL-12, and that the IgG2a subclass (a Th1-like antibody) response can be preferentially enhanced.

3.2. Proliferative response of splenocytes from mice vaccinated with B. mallei is enhanced by IL-12

We examined the effect of IL-12 on the proliferative response of splenocytes from mice vaccinated with *B. mallei*, *B. mallei* plus IL-12, or only IL-12. We saw a 5- to 24-fold increase in the amount of [3H]TdR incorporated when we compared *B. mallei* stimulated splenocytes with the respective unstimulated splenocytes from all groups of mice (Fig. 1). Comparing the amount of proliferation between splenocytes from mice that received non-viable *B. mallei* only (Fig. 1, group 2) and splenocytes from mice that received *B. mallei* and the least amount of IL-12 (0.1 μg) (Fig. 1, group 3), there was a 1.24-fold increase in proliferation. There was a significant increase (up to 1.9-fold; $P < 0.001$) in proliferation by the splenocytes from mice that received *B. mallei* and increasing amounts of IL-12 (0.25–1.0 μg), when compared to the splenocytes from mice that received only *B. mallei*. Splenocytes from mice that received only IL-12 (0.25 or 1.0 μg) were not stimulated. IL-12, therefore, when included with non-viable *B. mallei* cells was able to enhance the proliferative response of splenocytes when compared to splenocytes from mice that received only *B. mallei*.

3.3. IL-12 can augment the expression of IFN-γ in response to B. mallei in splenocyte cultures

We further evaluated the effect of IL-12 on the murine cellular immune response to *B. mallei* by examining the expression of IFN-γ by splenocytes as representative of a Th1-like cytokine, and the expression of IL-4 and IL-10 by splenocytes as representatives of a Th2-like cytokine. We found approximately a 1.5-fold increase in the amount of IFN-γ produced by splenocytes from mice treated only with non-viable *B. mallei* compared to cells from mice that received

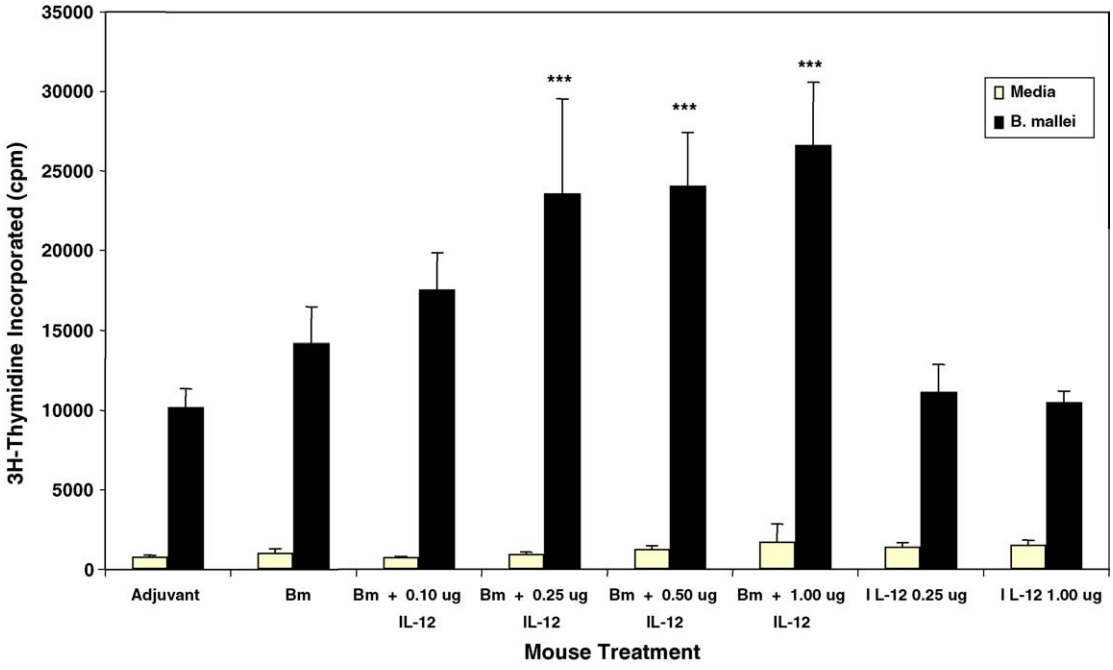

Fig. 1. Proliferative response of splenocytes from BALB/c mice that received *B. mallei* (Bm) with or without increasing amounts of IL-12. Mice were vaccinated twice s.c. with adjuvant only, *B. mallei*, *B. mallei* and 0.1–1.0 μg of IL-12, or with IL-12 only. All groups received adjuvant. There were five mice per group, except the groups with only IL-12, where there were four mice per group, and spleens were processed as described in Section 2, 30 days after the second boost. The results are derived from two sets of splenocytes from each group, performed in triplicate, and reported as the mean of the amount of 3H-thymidine incorporated ± S.D. For each group of mice, the splenocytes were incubated with media only or with non-viable *B. mallei* cells (10 μg/ml). There were only significant differences in the amount of proliferation when comparing the amount between the groups of mice that received only *B. mallei* and those that mice that received *B. mallei* and 0.25–1.0 μg of IL-12 ($^{***}P < 0.001$).

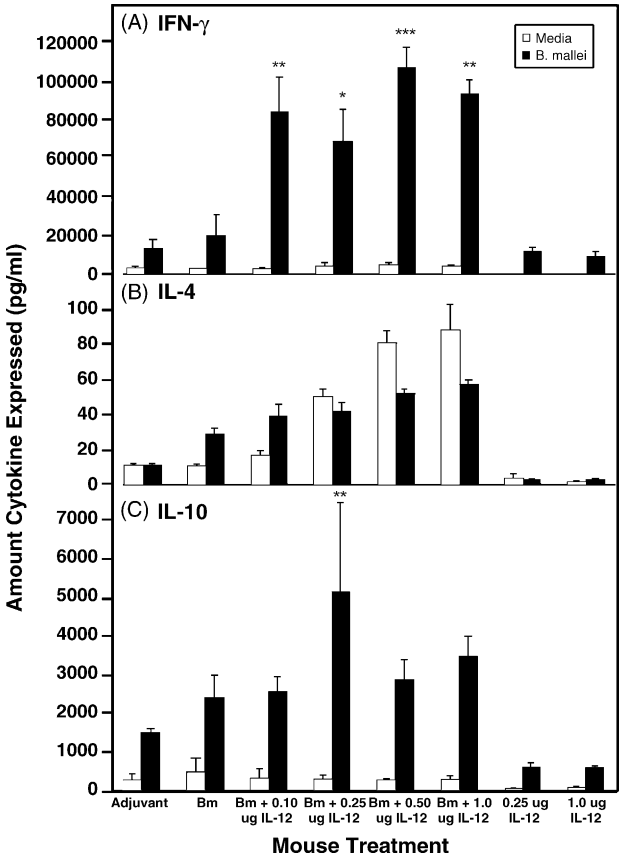

Fig. 2. Amount of cytokine expressed in the supernatant of splenocytes from mice that were vaccinated with adjuvant only, *B. mallei*, *B. mallei* and varying amounts of IL-12, or with IL-12 only. All groups received adjuvant. There were five mice per group, except the groups with only IL-12, where there were four mice per group, and spleens were processed as described in Section 2, 21 days after the second boost. Splenocytes incubated with media only or with non-viable *B. mallei* cells (10 μg/ml) for 45 h. The results are from two sets of splenocytes from each group. Cytokine analysis was performed in triplicate from each set of samples as described in Section 2. The results are reported as the mean of the amount of cytokine expressed ± S.D. (error bars). The amount of cytokine expressed for (A) IFN-γ, (B) IL-4, and (C) IL-10. There were significant differences in the amount of cytokine expressed by stimulated splenocytes, when compared with stimulated splenocytes from mice vaccinated with *B. mallei* only: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

only adjuvant (Fig. 2A). We found at least a 3.5- to 5.5-fold increase in the amount of IFN-γ in stimulated splenocyte cultures prepared from mice that received both *B. mallei* and IL-12 when compared with cultures of cells from mice that received only *B. mallei* cells (Fig. 2A; $P < 0.05$ to <0.001)). There was minimal expression of IFN-γ from splenocytes prepared from mice that only received IL-12 upon restimulation with non-viable *B. mallei* cells. We then examined the amount of IL-4 in the same splenocyte cultures and found that cells from mice that received *B. mallei* or *B. mallei* and 0.1 μg of IL-12 produced more IL-4 than the respective unstimulated splenocytes (Fig. 2B). However, splenocytes from mice that received higher amounts of IL-12 (0.25–1.0 μg), expressed less IL-4 than the same splenocytes that were unstimulated. When we examined the amount of IL-10 that was present under the same conditions (Fig. 2C), we found that with the addition of increasing amounts of IL-12 to the *B. mallei* cells in the vaccination treatment, there was a slight increase the amount of IL-10 produced by the *B. mallei* stimulated splenocytes, and a significant amount at 0.25 μg of IL-12 ($P < 0.01$). Splenocytes from mice that received only IL-12 produced similar or less amounts of cytokines than splenocytes from mice that received only adjuvant after restimulation. In conclusion, both Th1- and Th2-like cytokines are produced in response to non-viable *B. mallei*, but the inclusion of IL-12 appeared to generally augment the expression of the Th1-like cytokine more than the Th2-like cytokines.

3.4. IL-12 mediates partial protection from a lethal challenge in B. mallei vaccinated mice

Since IL-12 enhanced both the humoral and cellular immune response to non-viable *B. mallei*, we examined the ability of IL-12 to mediated protection from a lethal challenge of *B. mallei*. Mice were vaccinated with non-viable *B. mallei* and varying amounts of IL-12 (Table 1) and challenged with 143 LD_{50} of *B. mallei* i.p. and were observed for 21 days post-challenge (Fig. 3). No mice survived (0/10) from the group of mice that received adjuvant only (group 1), non-viable *B. mallei* only (group 2), or IL-12 only (groups 7 and 8). In these cases, the majority of mice died within 2–5 days after challenge. The mean survival time (MST) in days for these mice was 2.6 (±S.E. 0.2–0.4). Mice that received non-viable *B. mallei* and 0.1–1.0 ug of IL-12 (groups 3–6) were better able to survive the lethal challenge (2–6/10 survivors). There was a significant difference between the number of survivors in group 3 and 5 ($P < 0.05$) when they were compared to the number of survivors in group 2 (*B. mallei* only). Furthermore, there was a notable increase in the MST of mice in these two groups (13.6 ± 2.8–2.9). In spite of being able to survive the lethal challenge, however, the spleens of survivors from all groups were generally enlarged and all heavily infected with *B. mallei* (data not shown). It, therefore, appears that IL-12 can mediate partial protection, albeit not sterile, to mice that are vaccinated with non-viable *B. mallei* cells.

4. Discussion

In our previous study with capsulated or non-encapsulated, *B. mallei* cells as a vaccine, we found it offered no protection from a lethal challenge of live *B. mallei* cells [6]. We had also previously shown that there was minimal proliferation and IFN-γ expression by restimulated splenocytes from mice that received only *B. mallei* antigen, when compared to splenocytes from mice that received only the adjuvant [6]. With the addition of the cytokine IL-12 with the *B. mallei* cells in the present study, we were able to demonstrate limited efficacy against a lethal challenge in BALB/c mice. IL-12 affected both the overall murine humoral and cellular response to *B. mallei* cells. Similar results on the

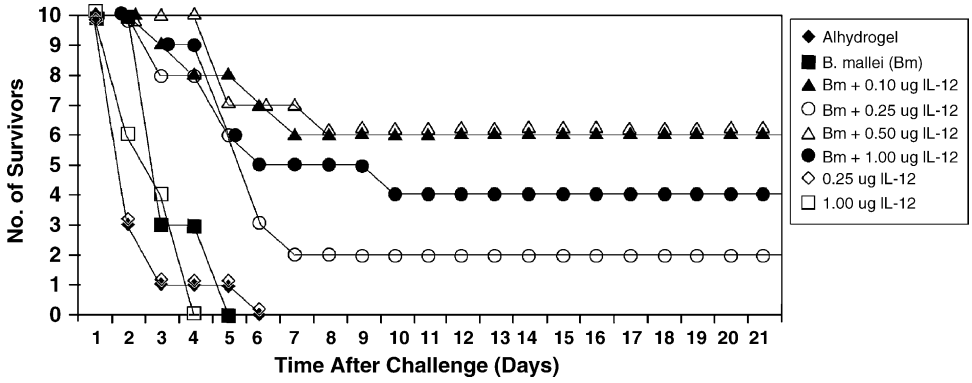

Fig. 3. Survival of mice vaccinated twice with adjuvant only, non-viable *B. mallei* cells with and without IL-12, or with IL-12 only after a lethal challenge. Mice were challenge (10 mice per group) i.p. with 1×10^8 *B. mallei* cells (154 LD_{50}) 21 days after the second vaccination boost and observed for 21 days. There was a significant difference in the number of survivors in groups of mice that received *B. mallei* + 0.1 ug of IL-12 ($P < 0.05$) or *B. mallei* + 0.5 ug of IL-12 ($P < 0.05$), when compared to the number of mice that survived in the group of mice that received only *B. mallei*. The results were compiled from two studies with 10 mice in each group, and similar results were obtained.

effect of IL-12 on the humoral and cellular immune response had been reported previously with a different antigen [34]. There was an enhancement of the IgG antibody titer and a differential effect on the production of Ig subclass IgG1 and IgG2a antibody levels with increasing amounts of IL-12. We also saw an increase in the proliferative response of splenocytes from mice that received both the antigen and IL-12, although we cannot completely discount some of the recall response to the presence of lipopolysaccharide (LPS) in our antigen preparation. In addition, we cannot rule out the possibility that IL-12 can potentiate the effect of LPS, since both can stimulate the innate immune system. At the same time, there was a differential augmentation of the expression of IFN-γ over that of IL-4 and IL-10 induced by IL-12. Our results suggest that the combination of enhanced antibody production and augmented expression of IFN-γ induced by IL-12 leading to a Th1-like immune response was responsible for the significant but partial protection of the mice from the lethal challenge, although this protection was not sterile. Further, the enhancement of the immune response to *B. mallei* by IL-12 depended on the presence of both the antigen and IL-12, since neither by themselves provided protection from the challenge. These results reflect the role of IL-12 as an immune modulator that affected the innate immune response to the antigen [7]. In addition, under the conditions used in the present study, we would not see an effect of IL-12 by itself because of the temporal aspect of the innate immune response.

In other animal model systems, the use of IL-12 as an adjuvant with a specific antigen induced an overall a protective response, which was characterized by the development of a Th1-like immune response [15,19,20,23,25,26]. Afonso et al. [19] found that the combination of leishmanial antigens and IL-12 led to the development of specific $CD4^+$ Th1-like cells that subsequently resulted in resistance to infection with *Leishmania major*. There was an increase in IFN-γ expression and simultaneous decrease in IL-4 production. The expression of IFN-γ appeared to be mediated by NK cells, as shown by an antibody that neutralized the cytotoxic function of NK cells. In a study with *Mycobacterium tuberculosis*, Lindbland et al. [20] showed that inclusion of IL-12 with other adjuvants and *M. tuberculosis* antigens induced a protective immune response, the extent of which was dependent on the concentration of IL-12. Mahon et al. [21] demonstrated that IL-12 increased the efficacy of a *Bordetella pertussis* acellular vaccine that was reflected in an increase in cellular proliferative response, IFN-γ expression, and protection. In the case with the intracellular bacterium *Listeria monocytogenes*, Miller et al. [23] used a heat killed cellular or soluble antigen with IL-12 to elicit a protective immune response. They saw an increase in IL-2 and IFN-γ expression in peritoneal T-cells, an increase in $CD3^+$ and αβ TCR^+ cell populations, and an increase in MHC-II expression and IL-12 expression by restimulated peritoneal macrophages. Wynn et al. in two separate studies [25,26] showed that IL-12 used either in a single or multiple vaccination regime with radiation attenuated cercariae of *Schistosoma mansoni*, a trematode worm, resulted in an increase in immunity to infection in mice. In both studies, a Th1-like humoral and cellular response was developed toward the irradiated cercariae and was dependant upon the administration of IL-12 with the antigen.

In studies with a closely related species, Santanirand et al. [37] found that resistance to infection by *B. pseudomallei*, the causative agent for melioidosis, in an outbred mouse model, was dependant on the expression of IFN-γ. The requirement for IFN-γ was shown by the administration of a neutralizing monoclonal antibody at the time of infection. Besides IFN-γ they found that resistance to infection also required the expression of IL-12 and TNF-α but not GM-CSF. In a follow up study Lertmemongkolchai et al. [38] found that NK and $CD8^+$ T-cells and not $CD4^+$ T-cells were the primary source for the production of IFN-γ in response to infection by *B. pseudomallei*. Neutralizing antibodies to IL-12 or IL-18

inhibited the expression of IFN-γ by NK and $CD8^+$ T-cells, and a monoclonal antibody to TNF-α had only a partial affect. Expression of IFN-γ by NK cells occurred quickly after infection (5 h) while $CD8^+$ T-cells expressed IFN-γ 15 h after infection. In addition, it was shown that the $CD8^+$ T-cells producing IFN-γ were predominately activated ($CD44^{high}$) in response to the infection.

In other studies with an infection model with *B. pseudomallei* with susceptible BALB/c mice and the relatively resistant C57BL/6 mice, it was found that there was an increase in both Th1- and Th2-like cytokines 24–36 h after infection. However, in BALB/c mice, the level of expression of some of these cytokines including IFN-γ was greater than in C57BL/6 mice, which was interpreted as a possible role for endotoxic shock and a cytokine-mediated immunopathology in the development of melioidosis [39]. It was further found that the mixed Th1- and Th2-like cytokine response induced by highly virulent and less virulent strains of *B. pseudomallei*, correlated with partial cross-protection between the two strains in mice previously immunized with the less virulent *B. pseudomallei* candidate vaccine strain [40].

In our studies with IL-12 and non-viable *B. mallei* cells, it appeared that the expression of IFN-γ was in part responsible for the partial protection from a lethal challenge. We would like to continue our studies to determine what type of immune cells are responding to the presence of IL-12 and the antigen. It is not clear at this time, if IFN-γ producing NK cells or $CD4^+$ or $CD8^+$ T-cells are responsible for the limited protection provided by IL-12 and *B. mallei* cells. Furthermore, we can evaluate the role of IL-10 in the limited protection we saw with IL-12, since IL-10 has been linked to suppression of Th1-like cytokine synthesis. Understanding the nature of the immune response under these conditions will help us design future vaccine candidates that could promote a more efficacious outcome in the prevention of glanders infections.

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